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A molecular method to quantify sex-specific consumption of Chinook salmon (*Oncorhynchus tshawytscha*) by Pacific harbor seals (*Phoca vitulina richardii*) using scat

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**A molecular method to quantify sex-specific consumption of Chinook salmon
(*Oncorhynchus tshawytscha*) by Pacific harbor seals (*Phoca vitulina richardii*) using scat**

By

Brittany Summer Balbag

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

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MASTER'S THESIS

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Brittany Balbag
February 19, 2016

**A molecular method to quantify sex-specific consumption of Chinook salmon
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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Brittany Summer Balbag
February 2016

ABSTRACT

Sex-biased predation may potentially skew sex ratios in adult populations, which may affect reproduction. Sex-biased predation by pinnipeds is of particular interest as it may impact fish populations of conservation and commercial interest such as salmon. However, sex-biased predation is difficult to measure in the wild and this is particularly true for marine mammals, since predation events in open water are often hidden from direct observation. Molecular scatology (genetic analyses of scat) has been used to non-invasively determine the proportion of prey items consumed in the diet, and it may be possible to determine sex-specific consumption of prey items using a similar approach. In this study, I develop a molecular method to measure the proportions of male and female Chinook salmon (*Oncorhynchus tshawytscha*) consumed by harbor seals (*Phoca vitulina richardii*) employing scat. By using QPCR, I established that the proportions of male and female Chinook DNA could be determined in a controlled mixed sample by measuring a y-linked marker, GHp-Y, in the sample in relation to a male control sample. I then applied the assay to harbor seal scat samples from haul-outs in the Strait of Georgia, Canada. Although the assay amplified in 83% of scat samples, 30% of scat samples quantified had an estimated male proportion > 1 . The lack of robustness of the assay might have been a result of contaminants in scat DNA extractions, which differentially impacted target genes. Lastly, using whole body tissue mixtures of males and females, I constructed a calibration curve to relate the DNA measurements of the assay to biomass proportions. The calibration curve was skewed by high male DNA density (presumably due to differences in gonad mass between sexes) precluding my ability to infer sex-specific consumption. Chinook populations return to rivers at different stages of reproductive development, and the tissue DNA density bias observed in this study

may only apply to certain prey populations in the field. Despite the DNA density male bias, the median estimated male proportion in scat samples was 0.31, which suggests that harbor seals are eating low amounts of male salmon and may have a bias towards females. However, further development of this approach is needed to make strong inferences about sex-specific consumption.

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INTRODUCTION

The impacts of predation on population dynamics can be complex and nuanced. In addition to reducing population size, predators may further influence population growth by impacting population demographics. For instance, they might display biases towards individuals of a certain sex, size, and/or age (Werner and Hall 1974, Hairston et al. 1983, Dickman et al. 1991, Gervasi et al. 2012, Hoy et al. 2015). Sex-biased predation has been observed in many predator-prey pairs such as bats and moths, lizards and kestrels, and weevils and plants (Acharya 1995, Marshall and Ganders 2001, Costantini et al. 2007, Boukal et al. 2008). Inherent differences in sexually-selected traits, size, or behavior between prey sexes may make one sex more conspicuous, easier to attack or handle, or of better food quality for predators (Hairston et al. 1983, Götmark et al. 1997, Zuk and Kolluru 1998). Depending on how many individuals are removed from the system, predators potentially may skew sex ratios in adult populations, which may affect reproduction and mate choice (Waples 2002, Boukal et al. 2008, Garner et al. 2010, Wedekind 2012).

Sex-biased predation must be observed in the wild in order to empirically determine its impact on natural populations. The majority of studies that have examined sex-biased predation in the wild have done so through directly observing predation events, collecting and sexing prey remains left in the environment, or examining sexually dimorphic structures in the gut (see review by Boukal et al. 2008). However, these approaches may not be feasible for many predator-prey interactions, which necessitate the development of new approaches that indirectly document sex-biased predation in the wild. This is particularly true for predation in marine mammals where predation events happen in the open waters where it is logistically difficult to observe these events and to collect prey remains.

In recent decades, understanding the diet of pinnipeds has become important as they prey on fisheries of conservation and commercial interest (Trites et al. 1997, Nash et al. 2000). In particular, the predation of Pacific harbor seals (*Phoca vitulina richardii*) on declining salmon populations, such as Chinook (*Oncorhynchus tshawytscha*) and Coho (*O. kisutch*) has become of interest to managers. Harbor seals typically target salmon in estuaries and rivers when they migrate to and from spawning rivers (Brown and Mate 1983, Wright et al. 2007). As salmon populations started to decline in the mid 1980s, harbor seal populations exponentially expanded leading to concern that harbors seals may impede the recovery of these populations (Bigg et al. 1990, Olesiuk 1993, NMFS 1997, Fraker and Mate 1999, Scordino 2010). In the Georgia Strait, researchers estimated that harbor seals consumed 2.8% of returning salmon in 1988 (Olesiuk et al. 1990). However, predation impact can vary by location and population; for example, in the Courtenay estuary off of Vancouver Island, harbor seals consumed approximately 46% of fall run Chinook salmon in 1989 (Bigg et al. 1990, Scordino 2010).

In populations that are highly impacted by harbor seals, it may be of interest to managers to know the proportion of male and female adult salmon consumed because understanding the factors that influence operational sex ratios is important in managing the productivity of salmon populations (DeWoody et al. 2010, Wedekind 2012). It is expected that sex ratios of salmon are 1:1 because salmon generally have XX/XY sex determination (Thorgaard 1977, Devlin et al. 2001). However, male-biased sex ratios in returning adults of Chinook and Coho populations have been observed, which may be due to sex-specific marine survival and sex differences in age at maturity (Trites et al. 1996, Spidle et al. 1998, Olsen et al. 2006). This is a concern to managers as females are the reproductively limiting sex. In

these populations, it would be important to know if sex-biased predation by harbor seals is contributing to these male-biased sex ratios in salmon.

Previous research on selective predation on salmon is limited. Sex- and size- biased predation on adult Sockeye salmon (*O. nerka*) in Alaska has been observed in brown bears (*Ursos arctos*) through measurements of carcasses (Quinn and Kinnison 1999, Ruggerone et al. 2000, Quinn and Buck 2001, Cunningham et al. 2013). The majority of these studies have demonstrated that brown bears select larger individuals over smaller ones and males over females. There may be a size component to this sex-biased predation as male sockeye salmon are typically larger than females (Young 2005). Brown bears may select for size because larger individuals may be more rewarding in terms of energy or more conspicuous to the predator (Quinn and Buck 2001, Cunningham et al. 2013). Adult Chinook salmon exhibit the strongest female-biased sexual size dimorphism in North America Pacific salmon (Young 2005). Returning female Chinook salmon may be bigger because females tend to return to rivers at later ages (Trites et al. 1996, Olsen et al. 2006). If size is a component in sex-biased predation on salmon, harbor seals may then selectively prey on Chinook female salmon.

Analysis of scat (scatology) may be a feasible way to indirectly address sex-biased predation in harbor seals. Scatology is already a popular approach to indirectly determine a marine mammal's diet, assuming that the prey species consumed will be represented in a scat sample. Scat is relatively easy to collect allowing researchers the ability to obtain sufficient samples sizes. Also, examination of scat is non-invasive and therefore resolves ethical concerns with disturbing rare and endangered/protected wildlife. Traditionally, the diet was determined through scat by examining the hard-parts of prey items (Olesiuk et al. 1990, Sinclair and Zeppelin 2002, Tollit et al. 2003, Trites and Joy 2005). In recent years, genetic

analysis of scat or molecular scatology has been used to identify prey species in the diet. Researchers identify prey items by amplifying prey-specific genetic markers by polymerase chain reaction (PCR) and sequencing PCR products to determine the species. This allows for greater confidence and taxonomic resolution in identifying prey species than hard-parts analysis (Deagle et al. 2005, 2009, Tollit et al. 2009). To determine the proportions of prey items in the diet, genetic techniques using quantitative PCR (QPCR) and next generation sequencing have been developed to quantify the proportions of prey DNA in scat (Deagle and Tollit 2006, Matejusková et al. 2008, Deagle et al. 2009, Bowles et al. 2011, Thomas et al. 2014). Captive feeding trials have been conducted to determine if the proportion of prey items measured by DNA reflects the proportions of prey items consumed (Deagle and Tollit 2006, Deagle et al. 2010, Bowles et al. 2011, Thomas et al. 2014). Some of these studies have shown that differences in tissue DNA densities and survival of DNA through digestion between prey species may bias estimates of diet composition. Depending on the degree of the bias, tissue and digestive correction factors can be used to improve estimates (Deagle et al. 2010, Thomas et al. 2014).

A number of genetic sex-determining assays have been developed for salmonids and these assays may be adapted for use with prey DNA from scat to determine sex-specific consumption of salmon in a harbor seal's diet. Because salmonids have XX/XY sex determination, an individual is determined to be either male or female by amplifying a genetic sex marker linked to the Y-chromosome by PCR (Du et al. 1993, Devlin et al. 2001, 2005). Genetic sex-determining assays have successfully been adapted to determine the proportion of male and female DNA in samples from human blood for forensic purposes, maternal plasma for fetal DNA analysis, and liver tissue after cell transplantation (Wang et

al. 2002, Horsman et al. 2006). Also, genetic sex-determining assays have been successfully applied to scat samples to determine the sex of predators (Reed et al. 1997, Ortega et al. 2004, Pilgrim et al. 2005, Matejusová et al. 2013). Thus, a genetic sex-determining assay could be applied to determine the proportion of male and female salmon DNA in scat. However, to the best of my knowledge, no one has established if genetic sexing assays can target prey DNA in scat.

In this study, I developed a method to measure the proportion of male and female Chinook salmon consumed by harbor seals in the Georgia Strait using genetic analysis of scat. First, I established whether a QPCR sex-determining assay developed for Chinook and Coho salmon (R. Devlin and D. Sakhrani¹, personal communication, May 23, 2014) could be used to determine the proportions of Chinook male and female DNA in a controlled mixed sample. This assay targets the growth-hormone pseudogene gene (GHp-Y), a y-linked marker. The GHp-Y serves as a reliable phenotypic sex-determining marker for Chinook populations in the Georgia Strait (Du et al. 1993, Devlin et al. 2001, 2005). Using QPCR, I measured the GHp-Y marker in relation to a male control sample and normalized this comparison using a reference marker, insulin-like growth factor 1 (IGF-1) to control for differences in DNA quantity so that the proportion of male DNA in a sample could be determined. I then assessed whether nuclear markers in the assay could be amplified and quantified in wild harbor seal scat samples to determine the proportions of male and female DNA in scat. This validation is important as prey DNA has experienced degradation from the digestive system and from environmental conditions making amplification of nuclear markers (as opposed to copy-rich mitochondrial markers) in prey DNA from scat potentially

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challenging. Also, DNA extracted from scat contains much non-target DNA and most likely contaminants that may negatively impact PCR amplification (Kohn and Wayne 1997, Wilson 1997, King et. al 2008).

However, the assay will only measure the ratios of male to female DNA in the prey DNA from a scat sample. Because males and females may differ in their DNA density, this proportion may not directly reflect the proportion of male and female biomass consumed by harbor seals. During migration to spawning grounds, the female gonad mass of Pacific salmon increases in size to 20% of body mass whereas in males it is about 3-5% (Hendry et al. 2000, Mann et al. 2009). Because the male gonad mass is smaller than the female gonad mass, I hypothesized that male whole-bodied tissues would be denser in somatic tissues than female whole-bodied tissues and as a result male tissues would be higher in DNA density than females tissues. As a result, female contributions may be underrepresented in samples based on DNA measurements. I, therefore, constructed a calibration curve to relate the DNA measurements of the assay to biomass proportions by applying the assay to known mixtures of male and female whole-bodied salmon.

MATERIALS AND METHODS

Determining whether the assay can measure male and female DNA proportions

To determine the proportion of male and female Chinook DNA in a sample, I used a Taqman® QPCR sex-determining assay to measure the difference in quantity of the GHp-Y marker between the sample and a male control and normalized this difference using a reference marker, IGF-1 (insulin-like growth factor 1). Primer and Taqman® probe sequences for the GHp-Y and IGF-1 are listed in Table 1 (R. Devlin and D. Sakhrani, personal communication, May 23, 2014). An 80 bp region in the GHp-Y sequence (between exon 5 and intron 5) was targeted to avoid amplification of growth hormone 1 (Genbank accession number S5087) and growth hormone 2 (Genbank accession number U28157) sequences. IGF-1 gene primers and probe target a 78 bp region and were determined from sequence U15960 (Genbank accession number). This assay was developed before the Taqman® QPCR sex-determining assay described in Nagler et al. 2004 (R. Devlin, personal communication, November 5, 2015). Primers were synthesized by IDT (San Diego, CA), and probes were synthesized by Applied Biosystems (Foster City, CA). Primer specificity was assessed using Primer Blast.

QPCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Standards and samples were run in 20 µl singleplex reactions in triplicate using a 96-well plate format (replicates were averaged together). Reactions contained 10 µl of Taqman gene expression mastermix (Applied Biosystems), 0.45 µM of IGF-1 primers, 0.9 µM of GHp-Y primers, 0.1 µM of IGF-1 probe, and 0.15 µM of the GHp-Y probe. Three no-template controls (template replaced with nuclease-free water) were included on every plate. Thermocycling conditions were as follows: 2 minutes at 50°C, 10

minutes at 95°C, and 40 cycles of 95°C for 15 seconds (denaturing) and 60°C for one minute (annealing and extension). Cycle thresholds (Ct) were calculated automatically by StepOnePlus™ Real-Time PCR System software (Applied Biosystems, Foster City, CA), but were visually checked to ensure that they were set in the linear phase of amplification.

To test if the assay could measure proportions of Chinook male and female DNA in controlled mixtures, I made a series of six mixtures of male and female DNA in the following proportions: 0:1, 0.2:0.8, 0.4:0.6, 0.8:0.2, and 1:0. Fin tissues were collected from juvenile Chinook salmon at the Centre for Aquaculture and Environmental Research (West Vancouver, BC, Canada). I extracted genomic DNA (gDNA) from fin tissues using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Concentrations of male and female DNA extracts from fin tissue were measured by fluorometry (Qubit® 2.0 Fluorometer, Invitrogen, Carlsbad, CA). The extractions were then diluted to 1 ng/μl and mixed to produce the proportions listed above. I diluted these mixtures 1:10 and 1:100 to assess whether the proportions could be measured at low DNA quantities. Markers were amplified in these mixtures using 2 μl of DNA template.

I determined the proportion of male DNA in samples using a relative quantification equation (Pfaffl 2001, Equation 1). This equation divides the fold change difference in GHp-Y between male calibrator and unknown by the fold change difference in the reference marker, IGF-1, to achieve normalization. Through normalization, the difference in DNA quantity between the two samples is controlled for and the proportion of male DNA can be determined.

$$RQ = \frac{(AF_{\text{GHP-Y}})^{\Delta Ct_{\text{GHP-Y}}}}{(AF_{\text{IGF-1}})^{\Delta Ct_{\text{IGF-1}}}}$$

where, AF = amplification factor

Ct = cycle threshold

ΔCt = Calibrator – Sample

(Equation 1)

Amplification factors (AFs) were determined by constructing standard curves. I created standard curves by diluting Chinook gDNA (quantified using fluorometry) 1:2 in nuclease-free water for both primer/probe sets (input range: 20 ng to 0.66 pg). Two microliters of template for each standard was used in the QPCR assays. Amplification factors were calculated using equation 2 (Pfaffl 2001)

$$\text{Amplification factor} = 10^{\frac{-1}{\text{slope}}}$$

(Equation 2)

Application of the assay to scat samples to measure male to female DNA proportions

Dr. Austen Thomas collected scat samples at haul-out sites near the following estuaries in the Georgia Strait: Cowichan, Fraser, and Courtenay from May through October of 2012 and April through October of 2013 (Fig. 1). The soft part scat matrix of the sample was homogenized and preserved in ethanol. He analyzed samples using next generation sequencing techniques for a molecular-based diet study. Prey species were identified by amplifying and sequencing a region of mitochondrial 16s ribosomal RNA. For the 2012 season, mitochondrial cytochrome oxidase II primers were used to distinguish between

salmonid species because the 16s was not able to distinguish between Coho and Steelhead (*O. mykiss*) sequences (Thomas 2015). These primers were not used for 2013 samples because it was found that the steelhead component of the diet was quite small, and consequently, the expense of using these additional primers was not justified for 2013 samples. Because IGF-1 primers used in this study were not salmonid species-specific as indicated by PrimerBlast (see Results) and GHp-Y primers amplify in Coho, I selected samples where the percentage of salmonid assigned sequences was $\geq 95\%$ assigned to Chinook based on the next-generation sequencing data (Thomas 2015). Out of 304 samples that contained Chinook, only 69 were selected among all locations based on this criterion (Table 2).

DNA from scat samples from the Courtenay estuary in 2012 was extracted in the summer of 2013, and the remaining samples were extracted in the summer of 2015 (Table 2). For DNA extraction, a subsample of the homogenized mixture was centrifuged and ethanol was poured off. The sample was further dried in a SpeedVac (Svc-100h, Savant Instruments, Inc., Farmingdale, NY) to remove the remaining ethanol and weighed to produce 180-220 mg of scat material. I extracted DNA from all scat samples using the QIAamp stool kit (Qiagen, Hilden, Germany). Samples that were extracted in 2015 were lysed overnight in ASL buffer (Qiagen) at 4°C and incubated at 70°C for 5 minutes before DNA extraction to increase DNA yield. At most 10 samples were processed at a time and extraction blanks (no scat matrix added) were included with each set of samples being processed to control for crossover contamination. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) was used to determine the purity and concentration of all DNA samples. NanoDrop A260/A230 readings < 2 indicate contamination with salt and

carbohydrate impurities and A260/A280 readings < 1.8 indicate contamination with protein (Eldh et al. 2012). DNA yields ranged from 2 - 56 μg . A260/280 ratios ranged from 1.5 - 2.4 and A260/230 ratios ranged from 0.9 - 2.3. All extractions were stored in a -20°C freezer until further analysis.

It is important that primers are specific to the intended target because genetic material in scat DNA samples is a metagenome comprised of DNA of different organisms. I tested the QPCR assay in DNA from harbor seal and the following non-salmonid fish species that represent fish taxa in the diet and are closely related to salmonids: Pacific cod (*Gadus macrocephalus*), dover sole (*Microstomus pacificus*), herring (*Clupea pallasii*), and Pacific rockfish (*Sebastes alutus*). Tissue samples from harbor seal were collected by Harriet Huber at the National Marine Mammal Laboratory, and I obtained tissue samples for the fish species from a local seafood store. DNA was extracted from these samples using the Qiagen DNeasy Tissue Kit, and I used 2 μl of DNA template for the QPCR assays. I further tested the assay in 15 scat samples (6 μl of template per sample) for which the sequencing of prey DNA did not yield any salmonid sequences (Thomas 2015). To further verify specificity, PCR products for 11 samples were run on 4% high-resolution agarose (Amresco, Solon, OH, USA) gels to ensure that the correct size marker was amplified. Lastly, a PCR product for each marker amplified in scat was inserted into a vector (pGEM[®]-T Vector Systems, Promega, Madison, WI, USA) following the manufacturer's protocol and sequenced in both directions using Sanger sequencing by Georgia Genomics (Athens, GA, USA). The sequences were aligned with a Chinook GHp-Y clone sequence (R. Devlin, personal communication, April 25, 2014) and a Chinook IGF-1 clone sequence (Genbank accession #: OTU14536) respectively. Pairwise alignments were created in Geneious v7.0.4.

The GHp-Y and IGF-1 markers were amplified in scat samples using 6 μ l of DNA template. I measured the proportion of male DNA in these samples by using the relative quantification calculation (equation 1) described above. Because markers may amplify differently in scat samples versus purified salmon genomic DNA, I compared a standard curve for both markers where salmon DNA was diluted in scat DNA extract to one where salmon DNA was diluted in water 1:5 (input: 100 ng to 0.032 ng). Because of differences in standard curves between the two types of dilution, I used AFs calculated from standard curves made from dilutions in scat extract. I used a single male Chinook salmon fin tissue DNA sample (s. above) diluted in the scat DNA extract as a calibrator. This scat DNA used for dilution was empirically tested to be negative for both markers using the QPCR assay.

I considered a sample to be positive for a marker if amplification ($C_t < 40$) was observed for all three replicates. The limit of quantification corresponded to a C_t value of 33 for both markers based on standard curves (Shipley 2013). Samples that do not amplify within this limit are subject to stochastic variation and are difficult to quantify. Samples that had a C_t value of the reference target >33 were excluded from the quantitative analysis. A sample that had a GHp-Y C_t value of >33 was only included in the quantitative analysis if the ΔC_T value between the GHp-Y and reference was greater than 5. A delta C_T value of 5 corresponds to a 25 fold-change (assuming AF of 1.9); and therefore, the error of the GHp-Y will play a minimal role in the overall relative quantification calculation. If a sample was not positive for the GHp-Y marker the proportion of male DNA in the sample was considered zero.

Post-hoc analyses of relative quantification

Thirty percent of scat samples displayed unusual relative quantification (RQ) values that did not fall between 0 and 1 (see Results). Because aberrant results may be due to differences in AFs between calibrator and samples, I calculated AFs by using the DART-PCR program (Peirson et al. 2003, Adams 2006, Čikoš et al. 2007). The DART-PCR program calculates AFs for each sample based on the PCR amplification curve generated by normalized fluorescent data and compares AFs between samples to detect outliers (Peirson et al. 2003). If outliers were detected I omitted them from the analysis. The AFs among PCR replicates and samples were averaged for respective PCR runs and RQ values were re-computed (equation 1) using these averages so that RQ calculations better reflect the kinetics of the run (Peirson et al. 2003). To validate this method, I calculated AFs using data from a PCR run that was used to construct standard curves from dilutions of Chinook DNA and compared calculated AFs to those calculated from standard curves.

To further determine if unexpected results were due to technical limitations, three samples were re-extracted (following the same protocol for extraction as the initial extractions) and were measured by QPCR. The purity was also determined for these three re-extractions based on NanoDrop readings. In addition, to determine if non-specific amplification was an issue, PCR products for eight of these samples were run on a 4% high-resolution agarose gel to determine whether correct size PCR product was obtained.

I hypothesized that sex-specific trends in consumption should only pertain to adult salmon as sex-specific traits become most apparent in the adult stage. Thus, I compared re-calculated RQ values between scat samples classified as adult and juvenile. Life stage of the Chinook salmon consumed was estimated using the split sample frequency of occurrence

approach. Salmon vertebrate structures were classified as either being juvenile or adult based on width measurements. The proportion of Chinook sequences were assigned to either adult or juvenile based on the frequency of adult and juvenile vertebrate structures in the sample (Thomas 2015). Only four scat samples quantified in this analysis were observed to have both juvenile and adult structures. Three of these samples were > 70 % adult and were classified as adult samples, and the other sample was 69% juvenile and was classified as juvenile in order to compare the two life stages in our analysis (see data from Thomas 2015). Lastly, because of differences in reproductive maturity between fall and spring Chinook populations (see Discussion), I tested whether re-calculated RQ values between adult spring (April-July) and adult fall (August-November) samples were different with a Kruskal-Wallis test.

Relating male to female DNA proportions to male to female biomass proportions

To determine if measurements of the assay could be related to proportions of male and female biomass, I mixed male and female whole-bodied tissues. Adult Coho fish were collected from the Cowlitz salmon hatchery (Salkum, WA). I used Coho fish because it was not possible to obtain Chinook salmon during the time of the experiment. The assay amplifies in Coho DNA, and it was determined that the markers amplify in Coho DNA at the same rate as Chinook DNA (data not shown). Three fish of each sex were homogenized separately in an electric meat grinder (Commercial-Grade 1-1/2 hp, Cabela's, Sidney, NE) and then further blended in a bladed food processor (Ninja BL770, SharkNinja, Newton, MA). Six 4 gram tissue mixtures were prepared by subsampling from male and female homogenates in these percentages: 0:100, 20:80, 40:60, 80:20, and 100:0. These mixtures

were stored in 95% ethanol and tissues were further blended with a micro homogenizer (T-25 digital Ultra-Turrax, IKA, Wilmington, North Carolina) to create an ethanol-tissue mixture. Three independent mixture sets were made. For DNA extraction, a subsample of the homogenized mixture was centrifuged and ethanol was poured off. The sample was further dried in a SpeedVac (Svc-100h) to remove the remaining ethanol and weighed to produce 20-25 mg of material. I extracted DNA using the Qiagen DNeasy tissue kit and 2 μ l of DNA template was used in the QPCR assays. For each series of mixtures, the proportion of male DNA was measured relative to the 100% male homogenate sample using equation 1.

RESULTS

Determining whether the assay can measure male to female DNA proportions

Based on standard curves, the amplification factor for both the GHp-Y and reference gene in salmon genomic DNA was 1.92 (Fig. 2), which is within the accepted ranges of 1.90 - 2.10 (Taylor et al. 2010). There was a strong linear relationship between the relative quantification (RQ) value and the proportion of male DNA in a sample at 2 ng (r^2 :0.99) and 0.2 ng (r^2 :0.98) gDNA (Fig. 3). The error of the assay in terms of quantifying the male percentage for all mixtures based on RQ values ranged from 5-10% for 2 ng input and 3.2 - 11% for 0.2 ng input (error: |% male in sample - % male measured|). This relationship was not maintained at a gDNA input of 0.02 ng (r^2 :0.5) (Fig. 3). Ct values at this input were beyond the limit of quantification determined by standard curves.

Application of assay to scat samples to determine male to female proportions

In terms of the specificity of the assay, Primer Blast predicted that the GHp-Y primers would not amplify in any sequences in the Genbank database, but that the IGF-1 primers would amplify in other salmonids. The assay did not amplify ($Ct > 40$) in the seal DNA tissue and in any of the extractions of non-salmonid fish species tested. Also, the assay did not amplify in samples that lacked salmonid species based on the next generation sequencing data (Thomas 2015). Agarose gels indicated that the correct size PCR products were amplified in scat samples (Fig. 4). Sequencing further confirmed that correct PCR products were amplified. The pairwise alignments indicated that the sequence of the PCR product had 98% identity to the reference sequence for both genetic markers (Table 3).

The IGF-1 marker amplified in 83% of samples (n= 57), and GHp-Y marker amplified in 61% of samples (n=42). Of the samples in which the IGF-1 and GHp-Y marker amplified in, the marker amplified within the limit of quantification in 82% (n=47) and 79% (n=33) of samples respectively. Sixty-four percent of all samples (n=44) were included in the quantitative analysis (Table 4).

Dilutions of Chinook genomic DNA in scat DNA extract produced higher amplification factors (1.99 for GHp-Y and 1.97 for IGF-1) than in water (1.92 for GHp-Y and 1.91 for IGF-1; Fig. 5). For scat samples, the median RQ was 0.35 (range: 0 - 8.0) based on standard curve amplification factors calculated from DNA diluted in scat. Thirty-one percent of samples had relative quantification (RQ) values > 1 (Fig. 6). PCR products for eight samples with RQ values > 1 were run on a gel and PCR amplicons were obtained that matched the predicted size under specific amplification.

Average amplification factors calculated by the DART-PCR program ranged from 2.02 - 2.12 for the GHp-Y and 1.94 – 2.00 for the IGF-1. The DART-PCR program identified 6 outliers in terms of amplification rate. After removing these outliers, the median RQ was 0.31 and 30% percent of samples had RQ values > 1 (range: 0 - 8.2; Fig. 6). Considering only values from 0-1, the median RQ was 0.015 (Fig. 7). The RQ values for juveniles (median=2.21, n=6,) were higher than the RQ values for adults (median=0.31, n=33; Fig. 8), but this was not significant ($p > 0.1$, Kruskal-Wallis t-test). Based on adult samples, RQ values for fall season (median=0.49, n=22) were significantly higher than the RQ values for spring season (median=0.00, n=11) ($p < 0.01$, Kruskal-Wallis t-test; Fig. 9).

Three samples that had RQ values of 7.02, 4.88, and 1.11 and were not identified as outliers, based on their amplification factor by the DART-PCR program, were re-extracted

and quantified. The RQ values for the re-extracted samples decreased to 0.80, 1.41, and 0.16 respectively. Also, the re-extractions had higher A260/A230 readings (2.51, 4.68, and 1.66) than the initial extractions (1.44, 0.9, and 1.4). A260/A280 readings of the re-extractions (2.14, 2.21, 2.07) were similar to the initial extractions (2.08, 2.38, 2.03).

Determining whether RQ values can be related to male to female biomass

Average RQ values for biomass mixtures ranged from 0.83 to 1.02 in order of increasing male biomass (Fig. 10). The assay overestimated male contributions 20 - 62% (Fig. 10). DNA extraction yields from 100% male homogenates (yields: 7.2 - 8.4 µg) were 16.8 - 19.5 times greater than yields from 100% female homogenates (0.42 – 0.50 µg).

DISCUSSION

To the best of my knowledge, this is the first study to directly target a sex-specific marker in prey DNA from scat. Only one other study reported amplification of a deer (prey) sex-specific marker while testing a genetic sex-determining assay developed for brown bears (*Ursus arctos*) for non-specific amplification in scat samples (Murphy et al. 2003). Typically, diet studies using molecular scatology target mitochondrial or multi-copy genes in prey DNA as opposed to single/ low-copy genes because the prey DNA is degraded and in low concentration in gut or scat samples (Zaidi et al. 1999, Deagle et al. 2006, Tollit et al. 2009). Despite issues with DNA degradation, I was able to amplify low-copy nuclear markers with relatively high success using QPCR in wild samples. To my knowledge, this may be the first study to achieve such success rates. QPCR is especially effective for analysis of degraded and low copy template as it is a sensitive technique that can quantify tens of copies of genes. Also, QPCR generally targets a short genetic marker (~80 bp), which improves the chances of amplification in degraded DNA (Deagle et al. 2006).

Although the QPCR assay was sensitive enough to estimate the proportion of male and female salmon in a controlled mixed sample (Fig. 3), it is unclear whether the assay can reliably estimate the proportion of male and female DNA in scat samples. Because the fold-change in the GHp-Y marker is normalized, ideally RQ values should fall between 0 and 1. Given that the assay can measure the proportion of male DNA with at most an 11% error, RQ values around 1.11 would be expected. However, 31% of samples had RQ values > 1.11 (Fig. 6).

A potential explanation for these values may be that the GHp-Y marker was amplifying in non-target (non-salmonid) DNA (note that only samples in which Chinook

salmon made up at $\geq 95\%$ of the salmonid sequences were analyzed). I was not able to test the assay in every fish species that may occur in a harbor seal's diet, but the species that I tested represent taxa that are common in the diet and closely related to salmonids. These taxa are basal to (herring) and part of (sole, rockfish, and cod) the larger clade (Euteleostei) containing the salmonids (Maddison and Schulz 2007). Because I observed no amplification in this phylogenetic distribution of prey fish, I believe that my primers are indeed specific to salmonids. In addition, PCR products for eight samples with RQ values >1 were run on a gel and correct size PCR amplicon was obtained, further suggesting that non-specific amplification was not a source of error in these samples.

Variation in amplification of the GHp-Y marker in male Chinook individuals might have been another confounding factor influencing relative quantification measurements. It is important that amplification between male individuals is consistent as samples are measured in relation to one male sample. One study reported variation in amplification of the GHp-Y marker in male Chinook individuals in one Columbia river and one Yukon river population (Nagler et al. 2004). Variation in amplification between males in certain populations may be due to sequence variation in GHp-Y or due to multiple copies of the gene (Devlin et al. 2005, Muttray et al. 2015). However, another study found variation in amplification in the GHp-Y to be low among individuals (Devlin et al. 2005). Researchers observed weak amplification in the marker in 25 (14%) individuals across seven populations in the U.S. and Canada mostly associated with one population in central British Columbia (Kitimat River). The marker did not weakly amplify in populations near the locations examined in this study. These results suggest that variation in amplification may occur only in some populations and not at a wide-scale. This is further supported by another large-scale study that showed that

the growth hormone pseudogene is a relatively conserved sequence among Chinook populations in the US and British Columbia (Muttray et al. 2015).

The more likely explanation for my abnormal results is sample contamination. Scat samples contain contaminants from the digestive system and the environment that may negatively impact amplification in terms of sensitivity and kinetics known as PCR inhibitors (Kohn and Wayne 1997, Wilson 1997). Although the DNA extraction technique was specifically developed to remove contaminants from DNA samples from feces, co-extraction of PCR inhibitors can still occur (King et al. 2008). NanoDrop readings indicated variation in contamination across samples, indicating that the DNA extraction technique I employed was not consistent in removing impurities (see Methods). Many samples had A260/A230 readings below 2, indicating contamination of salt and carbohydrate impurities, and A260/A280 readings below 1.8, indicating contamination of protein content (Eldh et al. 2012). PCR inhibitors may cause variation in amplification between samples and calibrator which is problematic as the relative quantification analysis assumes amplification is consistent (Ramakers et al. 2003, Brankatschk et al. 2012). One piece of evidence that supports this notion is that RQ values of three samples substantially changed after re-extraction. Also, the re-extractions appeared to be less contaminated as they had higher A260/230 readings. It should be noted that the PCR technical replication among re-extractions was consistent and that the same calibrator was used for the initial extractions.

Often inhibitors negatively impact the amplification rate by binding to Taq polymerase or blocking/competing with reagents (Opel et. al 2009). Measurements of amplification factors based on standard curves and the DART-PCR program suggest that these markers amplify with a higher rate in scat than purified fish genomic DNA (Fig. 5).

Amplification rates in DNA from environmental samples have been found to be higher than in pure standards (Callbeck et al. 2013). The effect of background DNA in scat samples may be similar to the effect of adding carrier DNA (tRNA, salmon sperm) to PCR mixes to prevent template DNA from adhering to the tube (Adams 2006, Callbeck et al. 2013). The DART-PCR program also indicated that calculated amplification factors of samples and calibrators were mostly comparable. Removal of outliers, in terms of the amplification rate, did not substantially affect the median RQ. This suggests that unexpected results may not be due to differences in amplification rates between samples and calibrator. Inhibitors can impact PCR amplification in other ways besides amplification rates by binding to or interacting with DNA template. For example, humic acid, which is commonly found in seawater and soils, inhibits the PCR by binding to DNA template, and therefore decreases the amount of template. This inhibition is sequence-specific, therefore, inhibitors may differentially impact target genes, which may explain aberrant results observed in this study (Opel et. al 2009, Gentry-Shields et al. 2013). Based on the data, it appears that inhibitors may be negatively impacting the IGF-1 marker more than GHp-Y marker.

This study highlights that greater quality control is needed when quantifying nuclear markers in scat. Ideally, DNA extracts should be as pure as possible, but this can be difficult to achieve with environmental samples. Further purification may be possible, however, this may reduce DNA yield and thereby compromise amplification success in prey DNA from scat. The use of a spike-in control in PCR reactions is a common way to determine inhibition in samples. Typically, the spike-in control is an exogenous DNA template at a known concentration, which allows one to further determine whether all samples are amplifying consistently (Matejusová et al. 2008, Bustin et al. 2009). However, this approach has

limitations as inhibitors may act differentially on DNA templates (Opel et. al 2009). The better approach to determine inhibition is to create dilutions of the DNA extract. If a sample suffers from inhibition, Ct values will not decrease as expected with an increase in dilutions (Bustin et al. 2009). The downside of this approach is that it is laborious when working with many samples and not feasible especially in studies like this one where the target DNA occurs at such low concentration (King et al. 2008). In future development, optimizing this assay and testing it in samples where the proportions of male and female DNA are known will be needed in order to gain confidence in its robustness. The addition of a PCR enhancer or master mix developed to be tolerant to inhibitors may improve assay robustness. In molecular scatology studies bovine serum albumin (BSA) is commonly used to relieve inhibition (Kohn and Wayne 1997, Deagle and Tollit 2006, Matejusová et al. 2008, Murray et al. 2011). BSA was not used in this study because it hindered amplification in preliminary results; however, the concentration of BSA may need to be optimized.

Previous molecular diet studies in pinnipeds demonstrated that QPCR can produce consistent measurements of proportions of prey DNA in scat samples (Deagle and Tollit 2006, Bowles et al. 2011). These QPCR assays may be more robust than the QPCR assay used in this study due to differences in PCR conditions or dilution of DNA extract. Also, these studies target mitochondrial markers, which may be less susceptible to inhibitors. Mitochondrial markers are copy rich and so the template density in the PCR reaction may be great enough to overcome the effects of the inhibitor. It is also important to note that these studies collected samples of captive animals while this study used samples of wild ones. Captive and wild scat samples may differ in quality since wild scat samples are exposed to more environmental contaminants; and therefore, inhibition may be more of a problem in

wild samples. Captive and wild animals also differ in diet and inhibitors associated with some prey tissues may negatively impact PCR amplification (Murphy et al. 2003, Pearson et al. 2015). Supporting this point, researchers found inhibition in scat samples from field samples, but not in captive samples using an exogenous spike-in control (Deagle et al. 2006, Matejusová et al. 2008).

In addition to potential PCR inhibitor biases, differences in DNA density between male and female salmon present another bias, which makes it difficult to infer male to female biomass consumption based on DNA measurements. Based on assay measurements, male contributions were so highly over-represented in biomass mixtures that a usable calibration curve could not be established (Fig. 10) because assay measurements could discern differences in biomass mixtures only to a very small extent. Assuming there are no digestive biases, it would be hard to infer the proportions of male and female consumed in the diet based on the curve given the PCR error in the assay.

Because the RQ value is directly related to the proportion of male DNA in a fish sample, the calibration curve suggests that male whole-bodied tissues have substantially greater DNA density than female tissues. This was further supported by DNA yields from male and female homogenates. I initially suspected that female whole body tissues, would be less dense than male tissues due to differences in density between somatic and gonad tissues, but not to the extreme found in this study. Although it represents a small portion of the male salmon body, the male gonad mass may be highly DNA dense due to a large number of small sperm and this may be contributing to the extreme differential in DNA density between the sexes. One study found that sequence proportions were not related to biomass proportions in a tissue mix of three species. Species that had more muscle and protein content were highly

over-represented based on DNA measurements. More protein is likely to be associated with higher density of muscle tissues, which have higher levels of DNA. Interestingly, the differences in protein and red muscle content between the three species were not as large as one would expect to result in such large differences in DNA density (Thomas et al. 2014). This is consistent to what I found in this study in that differences in DNA density between sexes were larger than I expected based on differences in tissue composition.

In this study, I constructed biomass mixtures using reproductively mature male and female salmon with fully developed gonad masses. Because the DNA density bias between males and females may be due to differences in gonad mass size, determining the realistic importance of these biases in the field may be dependent on the reproductive stage harbor seals encounter salmon in estuaries. It is unknown at what level of maturity harbor seals encounter salmon, but it may depend on the population. Generally, populations of Chinook salmon are divided into two life history types: stream-type and ocean-type. Stream-type Chinook return to natal rivers months before spawning in early summer/spring (Healey 1991). In these populations, harbor seals may encounter females whose gonad masses are similar to males and so differences in DNA density between the sexes may not be substantial. Ocean-type Chinook, on the other hand, typically return in the fall and spawn shortly after returning to the natal river (Healey 1991). Therefore, harbor seals may be encountering females whose gonads masses are fully developed and so the observed biases may be relevant. Assuming there is no difference in sex ratio returns for spring and falls samples, one would expect the distribution of RQ values for the fall season to be different from the spring season in that higher RQ values would be observed in the fall because there is a greater chance of detecting male DNA because of high male tissue DNA density. I did find that RQ

values for the fall were significantly higher than spring, which may support this notion (Fig. 9).

Partial consumption of the salmon body may be another confounding factor in determining sex-specific consumption in harbor seals. In this study, I assumed that harbor seals consumed all the salmon body, or at the very least, partially consumed the salmon body in the same manner for both the sexes. However, it is possible that harbor seals display sex-specific partial consumption. One study found that harbor seals consumed most of the body of male salmon, but consumed mostly the egg mass of female salmon (Hauser et al. 2008). It is unknown how widespread this behavior is, but this may further exacerbate the bias towards males in DNA estimates and obscure the relative number of male and female salmon killed by harbor seals.

It is quite remarkable that despite the extreme DNA male bias and the potential inhibitor PCR bias that the median estimated male DNA proportion was 0.31 (Fig. 6), which is lower than I would expect. This suggests that harbor seals are eating low amounts of male salmon. I expected to observe sex-specific trends of consumption in adult samples, but not in juvenile samples as sexually distinct traits become apparent in adult stages. Based on the data, it appears that harbor seals are eating high amounts of juvenile male salmon (Fig. 8); however, given the error in DNA measurements in scat and the small juvenile sample set, it is difficult to determine if this is an actual trend. Considering only adult data, a median estimated male proportion of zero for spring samples suggests harbor seals are eating very low amounts of male salmon. Based on fall samples, if we were to assume that the male DNA bias is relevant, the data (median RQ= 0.49) would suggest that harbor seals are eating low amounts of male salmon (Fig. 9). It's important to note that the prey DNA in most of the

samples quantified was primarily composed of Chinook (see data from Thomas 2015).

Samples where the prey DNA was mostly Chinook were probably most likely able to meet the criteria of sample selection and amplify within the limit of quantification. These samples may represent foraging events where large adult Chinook salmon were consumed. Because of the size differential between the sexes this may have biased the data to reflect foraging events where female salmon were consumed. Overall, this molecular scatology approach needs further development (as discussed in Conclusions and Future Directions) in order to make sound inferences to sex-specific consumption.

CONCLUSIONS AND FUTURE DIRECTIONS

This is the first study to apply a genetic sex-determining assay to scat samples in order to determine sex-specific consumption of prey. The nature of this study was largely exploratory in order to determine if this approach was feasible. I amplified low-copy nuclear genes of the assay with relatively high success, which perhaps gives promise to the use of other low-copy nuclear markers in other diet studies using molecular scatology. However, further development of this approach is needed, as it appears that the assay is not robust enough to measure the proportions of male and female DNA in scat samples. Unusual results may have been due to the co-extraction of PCR inhibitors that differentially impact target genes in the assay. This should be further confirmed by testing the assay in scat samples with known proportions of male and female DNA. The addition of a PCR enhancer may relieve inhibition and improve assay robustness. The use of molecular scatology to quantify the diet is a relatively new approach that has only been validated in captive samples. This study suggests that further validation of this approach is needed especially in wild samples.

In addition, to further developing the assay's robustness, species-specific primers will need to be developed, which will allow this approach to be applied to a greater number of scat samples. A larger sample is needed in order for one to make strong statistical conclusions about the proportions of male and female salmon consumed in the harbor seal's diet. Due to sample selection, the prey DNA in most of the samples quantified in this study was mostly comprised of Chinook DNA. These samples may have represented foraging events where large adult Chinook salmon were consumed, which may have biased the data and my conclusions. Thus, further development is needed in order to make this approach less specific in terms of sample selection so that samples measured encompass a variation in

foraging events in the predator population. Practically speaking, it is also necessary to make species-specific primers as its unlikely researchers will have beforehand knowledge of salmon species in a scat sample.

Even if a robust assay could be achieved, extreme differences in DNA density between males and females found in this study brings into question whether the proportions of male and female salmon consumed can be estimated based on genetic analyses. DNA density biases between the sexes may be due to differences in the size and DNA density of the gonad mass. A better understanding of the differences in tissue composition between males and females during migration to spawning rivers and how this influences whole-bodied DNA density is needed. Chinook populations return to rivers at different stages of reproductive maturity and the tissue DNA bias may be relevant to certain populations. Despite the supposed male bias, the center of the distribution of RQ values for scat samples is lower than I would expect, which possibly suggests that harbor seals are eating low amounts of male salmon. However, more development of the overall approach is needed to confidently infer trends of male to female consumption. This includes obtaining accurate estimates of Chinook male to female DNA ratios in scat, applying this approach to a larger set of samples, and a better understanding of the relevancy of the tissue DNA bias in the field.

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TABLES

Table 1. Sequences for the growth hormone pseudogene (GHP-Y) and insulin-like growth factor 1 (IGF-1) primers and TaqMan® probes used in the quantitative PCR assay (R. Devlin and D. Sakhrani, personal communication, May 23, 2014).

Primer or probe	Sequence 5' -3'	Reporter	Quencher
Growth hormone pseudogene			
Forward primer	GATGACAATGACTCTCAGCATCTG	-----	-----
Reverse primer	GACCCAAAGATACGTCCAGGTT	-----	-----
GHP-Y probe	ATGCGGGAAC TAATG	FAM	Non-fluorescent
Insulin-like growth factor 1			
Forward primer	TGCGATGTGCTGTATCTCCTGTA	-----	-----
Reverse primer	CCTGTTGCCGCCGAAGT	-----	-----
IGF-1 probe	TCTCACTGCTGCTGTGC	VIC	Non-fluorescent

Table 2. Breakdown of the number of harbor seal scat samples assayed by location, year, and season: spring (April-July), and fall (August-November).

Location	2012	2013	Total	Spring	Fall
Courtenay	16	4	20	10	10
Cowichan	16	4	20	8	12
Fraser	17	12	29	13	16
Total	49	20	69	31	38

Table 3. IGF-1 (a) and GHp-Y (b) PCR product sequenced from sample Pv12477. The sequences were aligned with a Chinook GHp-Y clone sequence (R. Devlin, personal communication, April, 25, 2014) and a Chinook IGF-1 clone sequence (genbank accession #: OTU14536) respectively. The second row of the alignment is the consensus sequence.

a)

Identities = 77/78 (98%),
Positives = 77/78 (98%), Gaps = 0/78 (0%)

```
PV12477-IGF-1 T G C G A T G T G C T G T A T C T C C T G T A C C C A C A C C C T C T C A C T G C T G C T G T G C G T C C T A A C C C T G A C T T C G G C G G C A A C A G G
                T G C G A T G T G C T G T   T C T C C T G T A C C C A C A C C C T C T C A C T G C T G C T G T G C G T C C T A A C C C T G A C T T C G G C G G C A A C A G G
OTU14536      T G C G A T G T G C T G T G T C C T G T A C C C A C A C C C T C T C A C T G C T G C T G T G C G T C C T A A C C C T G A C T T C G G C G G C A A C A G G
```

b)

Identities = 80/81 (98%),
Positives = 80/81 (98%), Gaps = 0/81 (0%)

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PV12477GHp-Y G A T G A C A A T G A C T C T C A G C A T C T G C C C C C C T G C G G G A A C T A A T A C C A G A A C C T G G G G C G A A C C T G G A C G T A T C T T T G G G T C
                G   T G A C A A T G A C T C T C A G C A T C T G C C C C C C T G C G G G A A C T A A T A C C A G A A C C T G G G G C G A A C C T G G A C G T A T C T T T G G G T C
GHp-Yclone   G G T G A C A A T G A C T C T C A G C A T C T G C C C C C C T G C G G G A A C T A A T A C C A G A A C C T G G G G C G A A C C T G G A C G T A T C T T T G G G T C
```


Table 4. Breakdown of the number of harbor seal scat samples used in quantitative analysis by location, year, and season: spring (April-July), and fall (August-November).

Location	2012	2013	Total	Spring	Fall
Courtenay	9	2	11	5	6
Cowichan	8	4	12	3	9
Fraser	15	6	21	8	13
Total	32	12	44	16	28

FIGURES

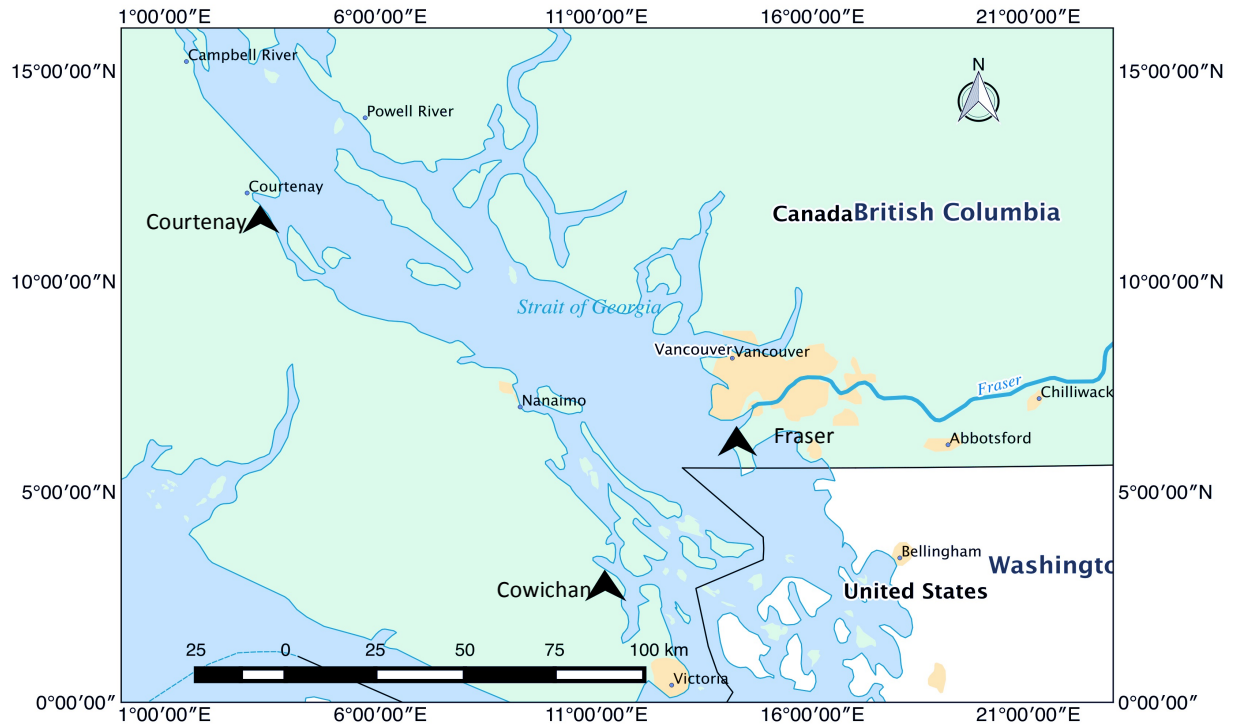


Figure 1. Locations (black arrows) of haul-out sites where harbor seal scat samples were collected. For details see Thomas (2015). Map created using Natural Earth data in QGIS.

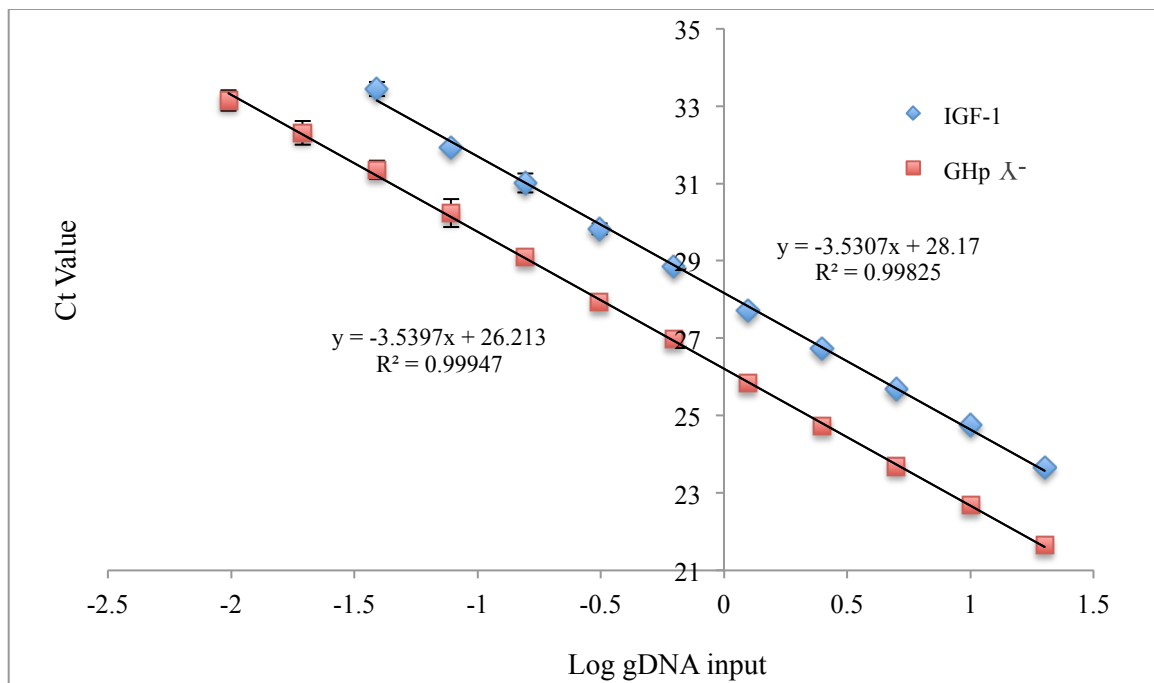
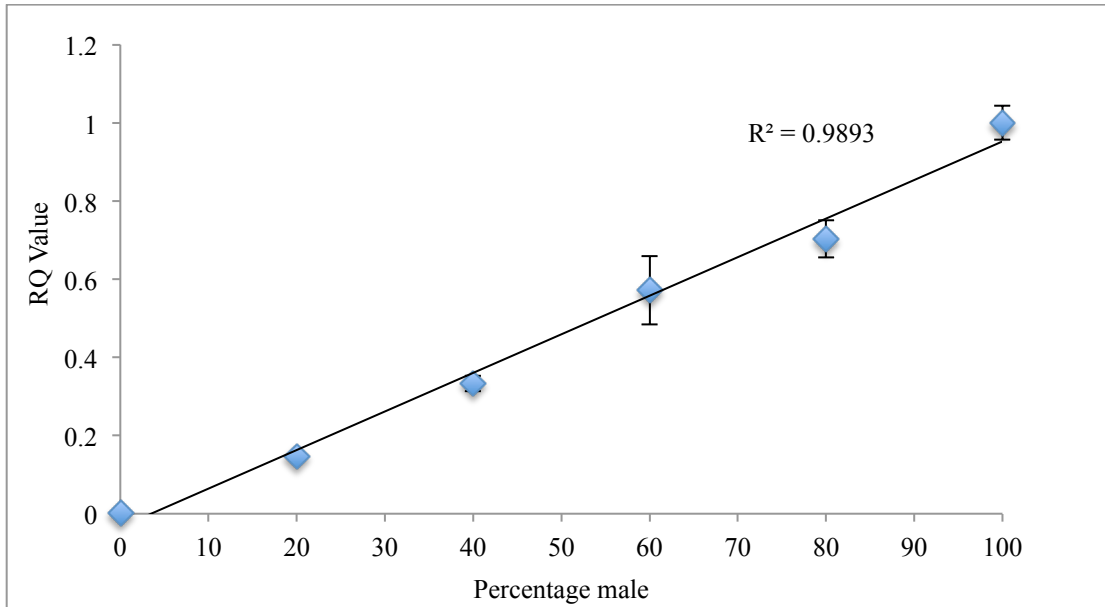
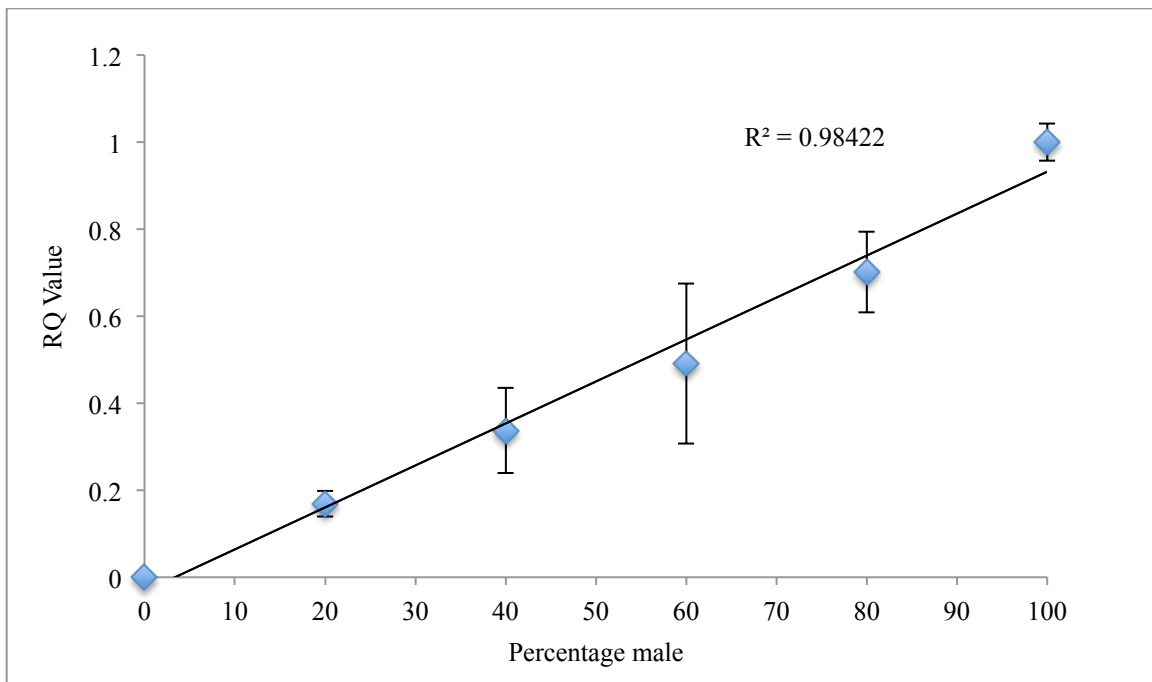


Figure 2. Standard curves for IGF-1 and GHp-Y primer/probe sets in salmon genomic DNA. Fish gDNA was diluted 2-fold (input range: 20ng to 6.6×10^{-4} ng). Error bars represent standard deviations of triplicate reactions.

a)



b)



c)

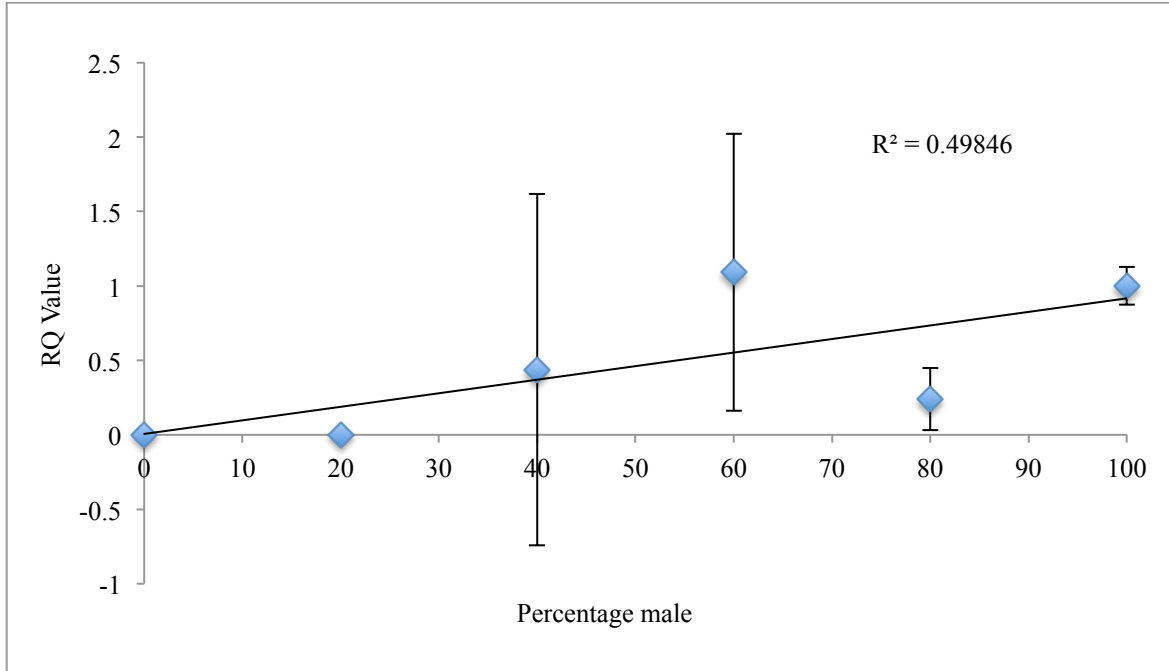


Figure 3. The relationship between RQ value and the percentage of male DNA in a mixed Chinook gDNA sample. The relationship is shown at different sample DNA quantities- a) 2 ng, b) 0.2 ng, c) 0.02 ng). Error bars represent standard error of triplicate reactions.

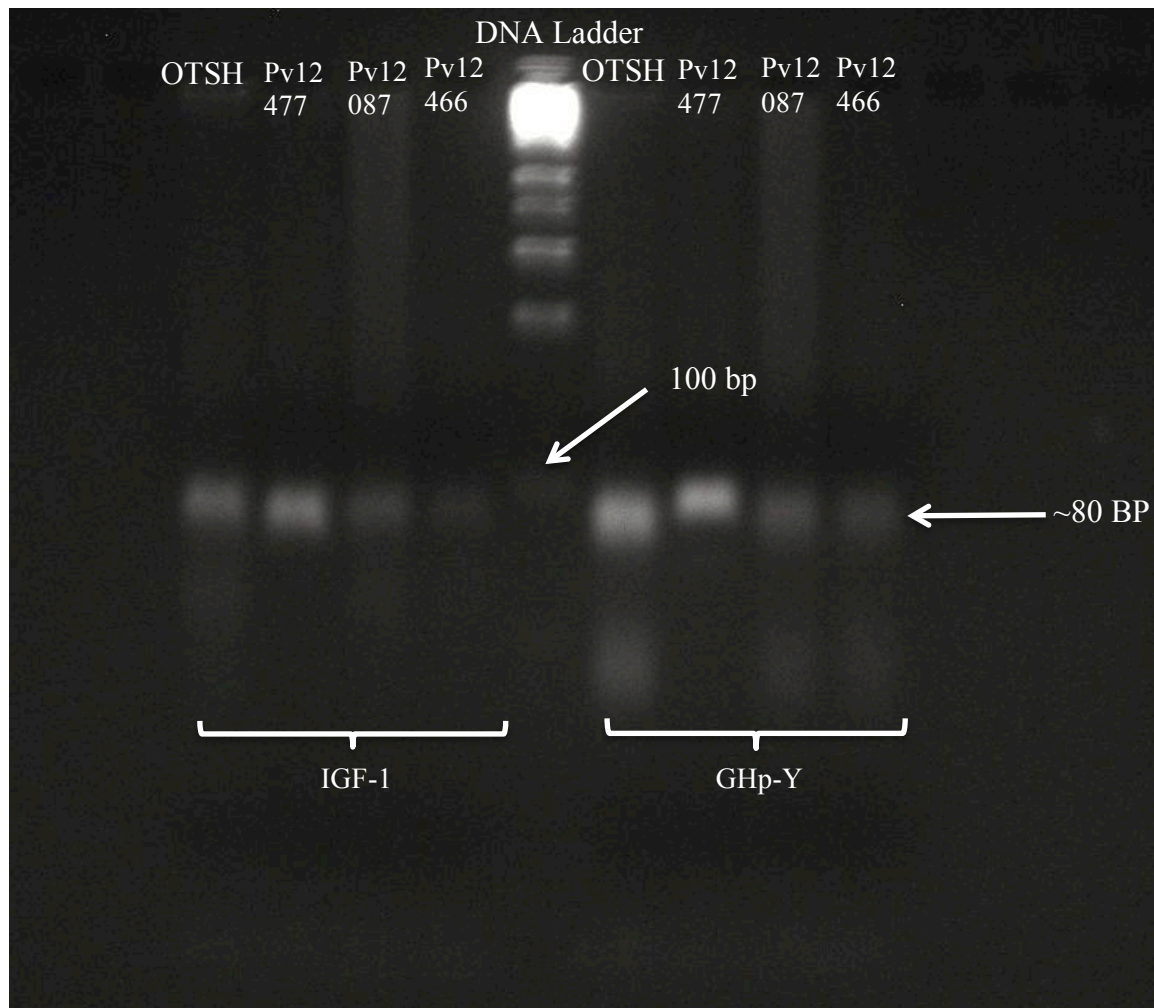


Figure 4. Amplified products in three scat samples and Chinook (OTSH) sample were run on a 4% agarose gel to confirm that products matching the predicted size of the IGF-1 (78 bp) and GHp-Y (80 bp) markers were amplified.

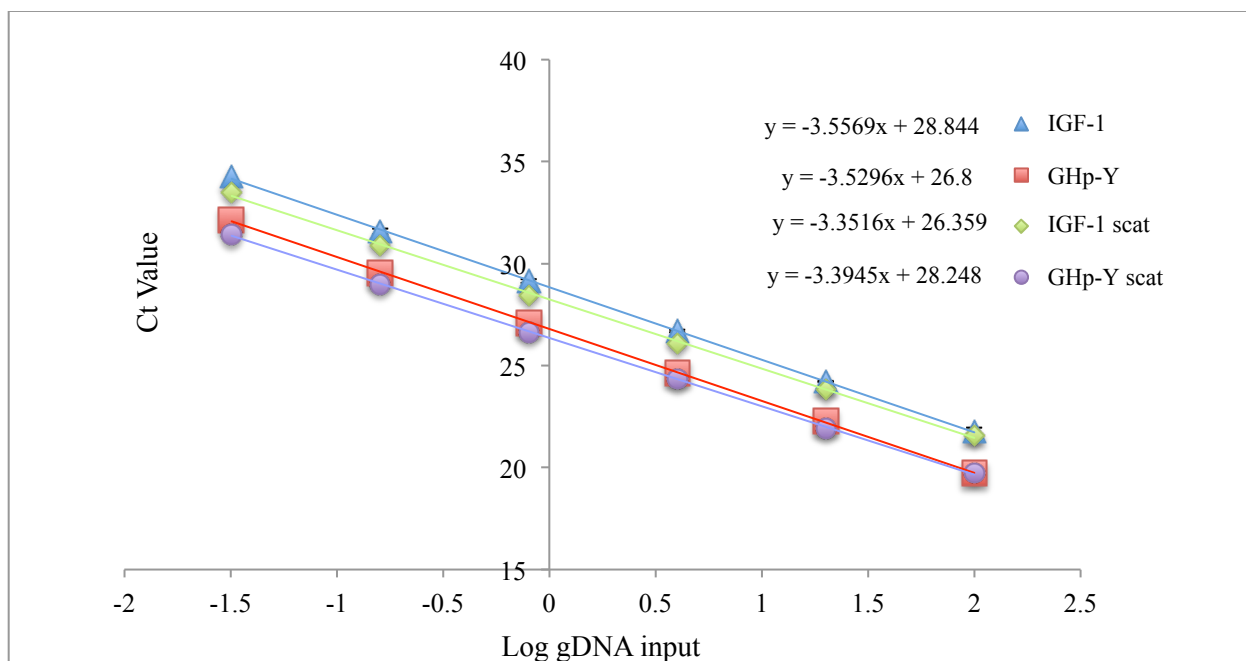


Figure 5. Standard curves for fish genomic DNA diluted in a 1:5 mixture of water and scat DNA extract (input: 100 ng to 0.032 ng) for both GHp-Y and IGF-1 primer/probe sets.

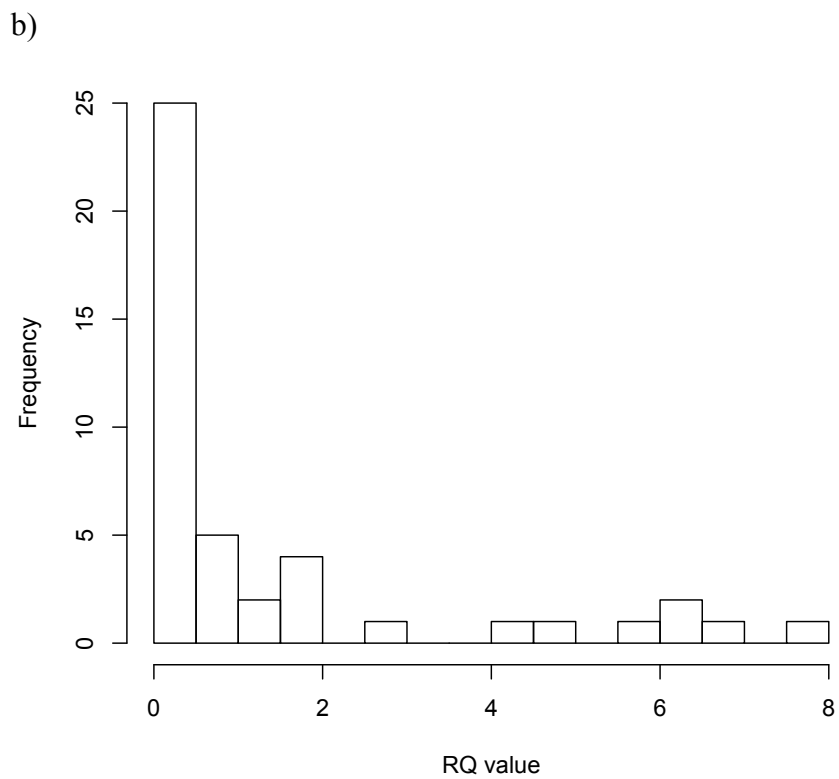
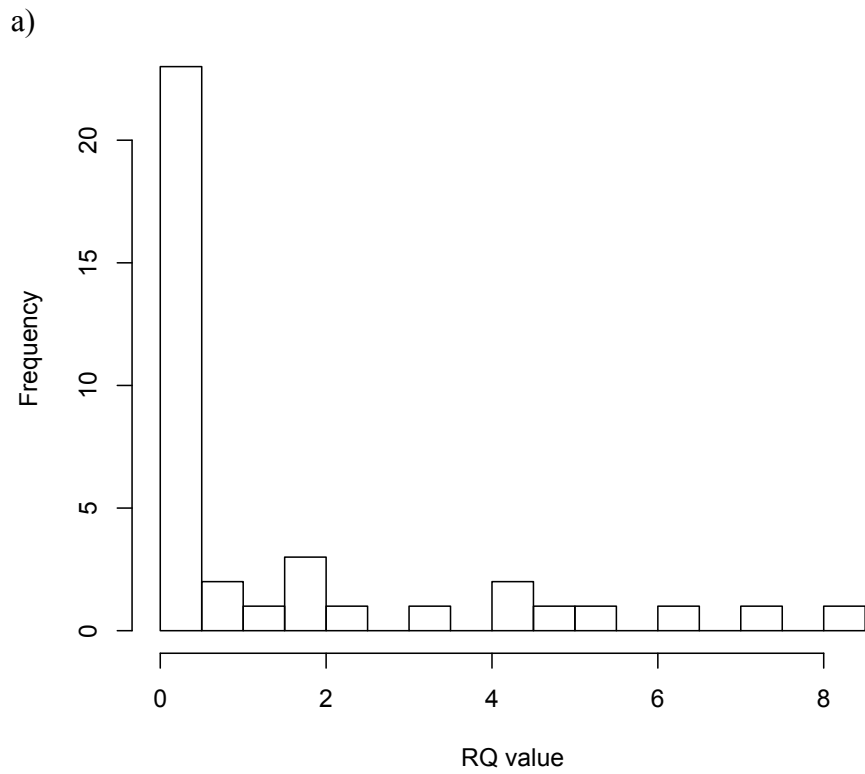


Figure 6. Histogram of RQ values measured in scat samples based on a) standard curve (n=44) and b) DART-PCR (n=38) amplification factors.

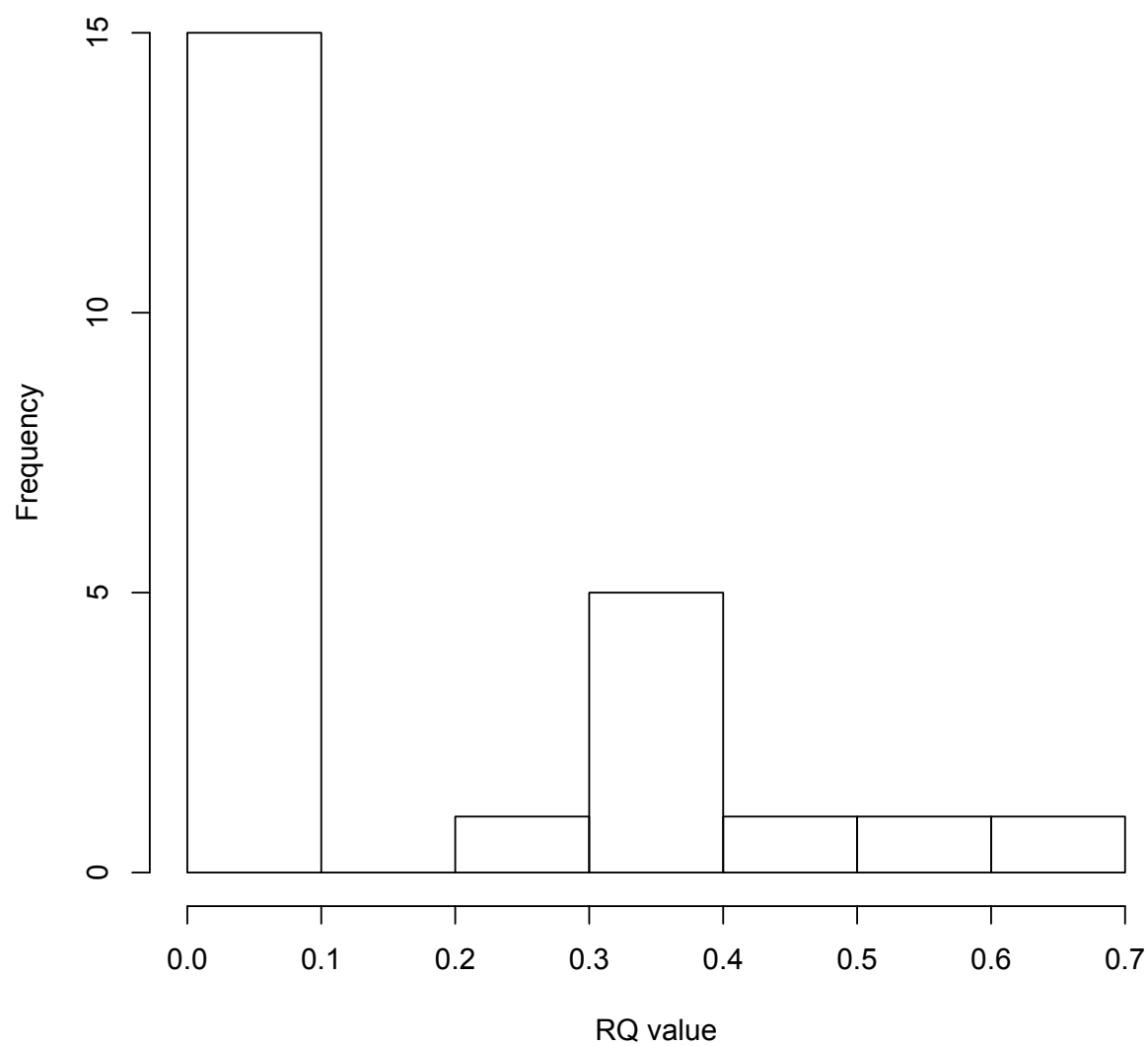


Figure 7. Histogram of RQ values measured in scat samples from 0 to 1 (n=25) based on the DART-PCR analysis.

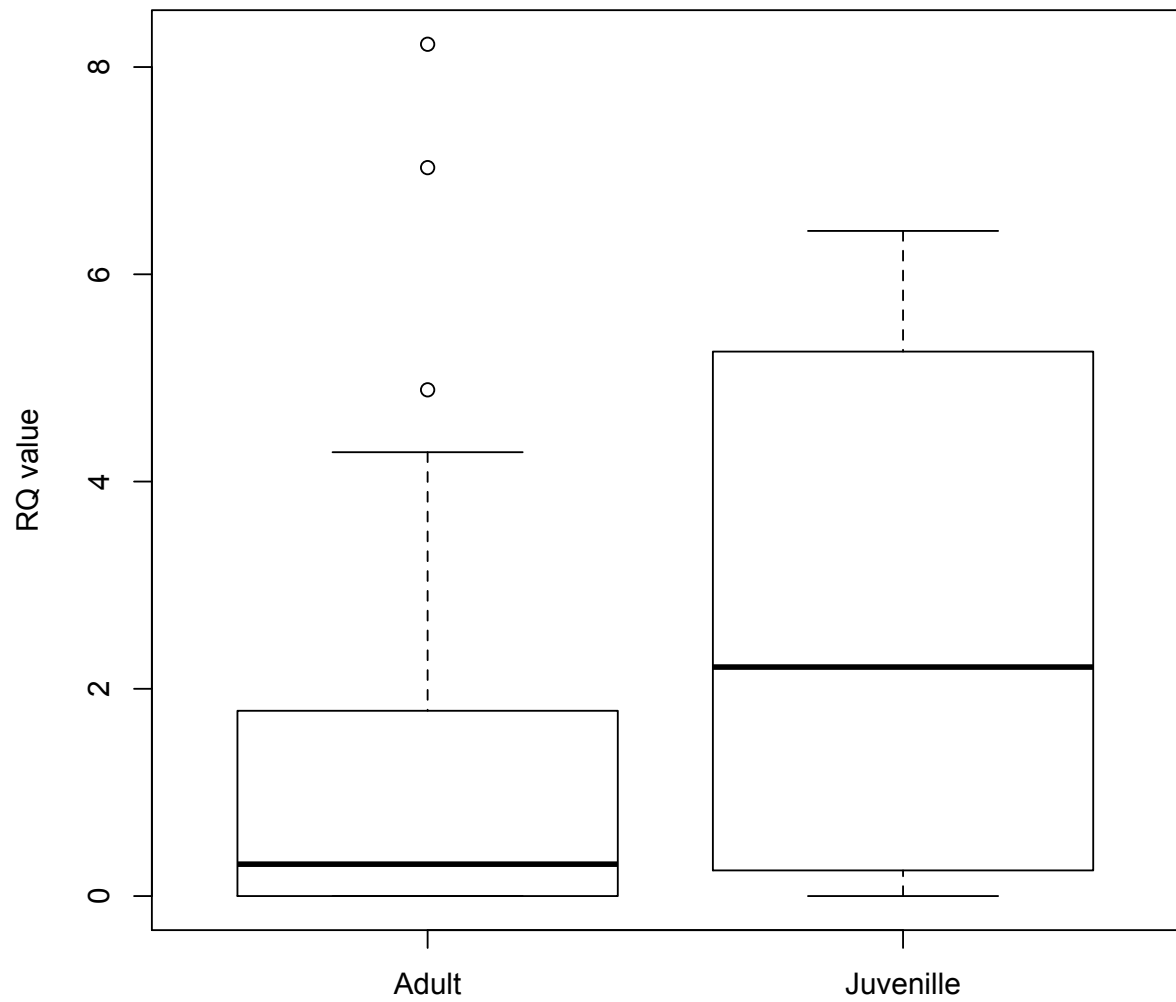


Figure 8. Distribution of RQ values for adult (n=32) and juvenile (n=6) scat samples based on DART-PCR analysis.

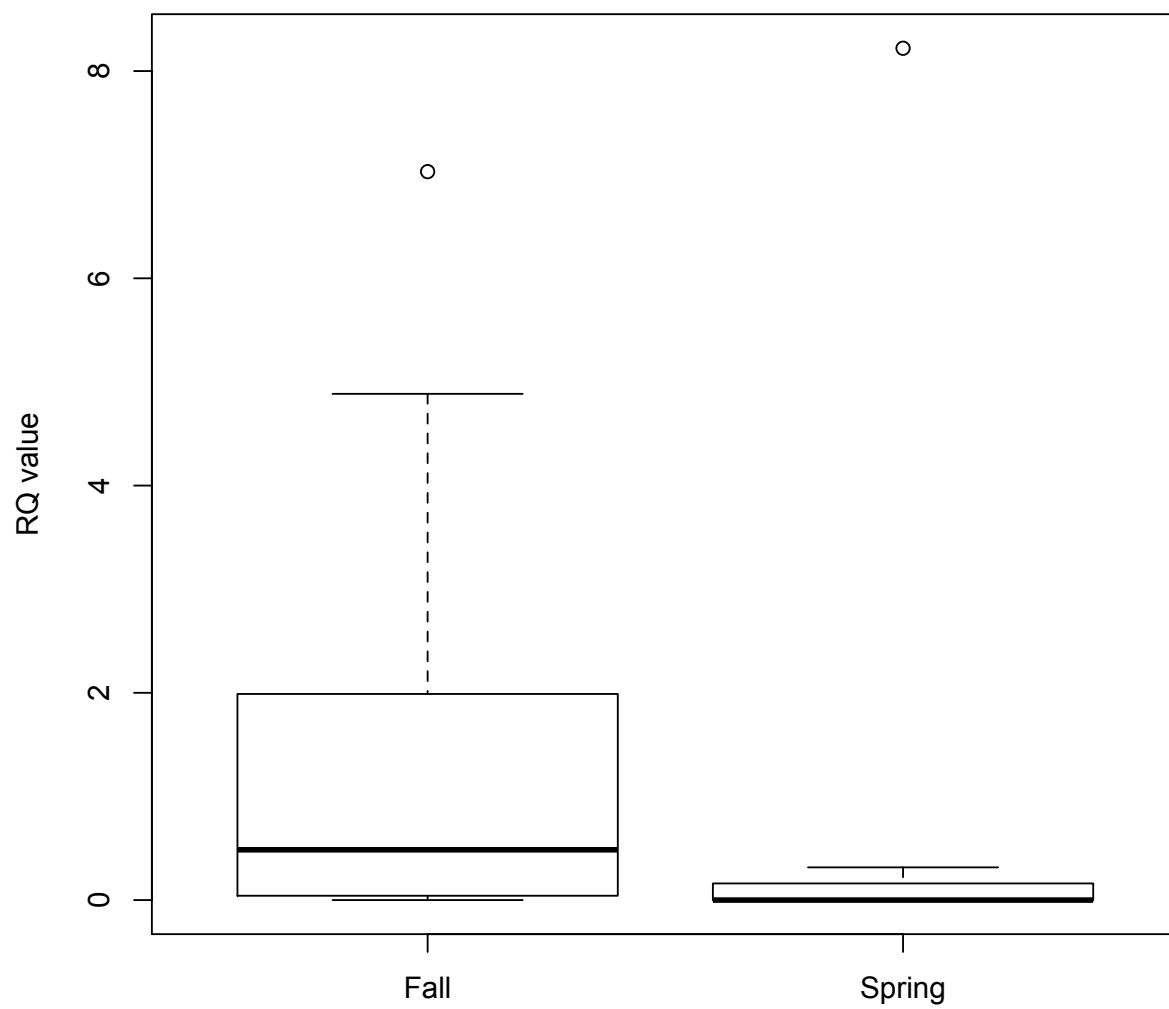


Figure 9. Distribution of RQ values for fall (n=22) and spring (n=11) scat samples based on DART-PCR analysis.

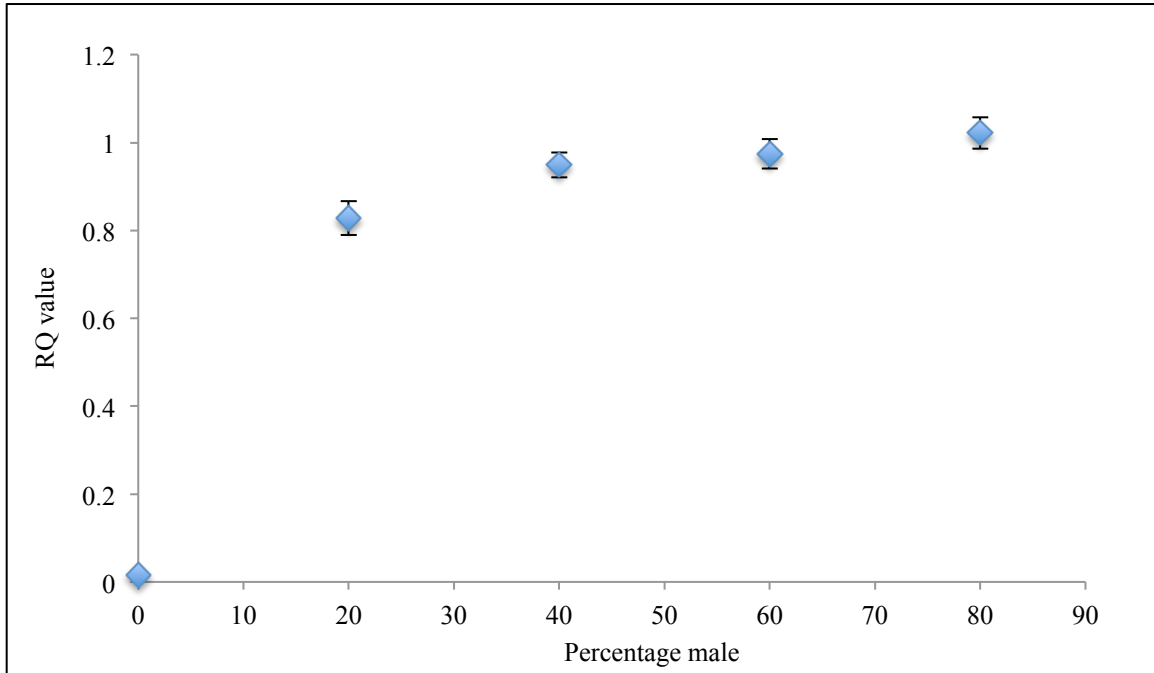


Figure 10. The relationship between RQ value and percentage of Chinook male biomass in a mixed biomass sample. Error bars represent standard error of three biomass mixture sets.